RyR1 and its protein ligands are often not stable in vitro and therefore difficult to study by single-particle cryo-electron microscopy (cryo-EM). The main goal of this study was to develop a procedure to cross-link protein ligands to RyR1 and visualize these complexes by single-particle cryo-EM. To test the cross-linking protocol we used the complex of FK506-binding protein and RyR1 (FKBP12:RyR1) as a model system, since the structure of FKBP12:RyR1 is known. Glutaraldehyde quantitatively cross-linked RyR1 subunits to each other and FKBP12 to RyR1 without damaging the ultrastructure. Cross-linked FKBP12:RyR1 was visualized in 2D averages, and was identical to that of previously published non-cross-linked FKBP12:RyR1. The effect of glutaraldehyde cross-linking on RyR1 structure was characterized using 3D single-particle cryo-EM and by [3H]ryanodine binding assay. Glutaraldehyde cross-linking preserved the gross morphology of RyR1, but induced minor structural changes at the cytoplasmic and transmembrane regions of RyR1. Glutaraldehyde cross-linking enhanced [3H]ryanodine binding to RyR1 by ~30%. Based on these results we propose that cross-linking RyR1 subunits by glutaraldehyde locked RyR1 in an open-like conformation state.

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FRET-based Structural Measurements of the Type 1 Ryanodine Receptor using Site-Specific Fluorophore Labeling to Tetracysteine Motifs

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The type 1 ryanodine receptor (RyR1) is an intracellular Ca^{2+} release channel that mediates skeletal muscle excitation contraction coupling. While the overall shape of RyR1 has been elucidated using cryo electron microscopic reconstructions, fine structural details remain elusive. To better understand the structure of RyR1, we have previously used a fluorescence resonance energy transfer (FRET)-based method using a fused green fluorescent protein (GFP) donor and a fluorescent acceptor, Cy3NTA that binds specifically to short poly-histidine 'tags' engineered into RyR1. However, the large size of the GFP fusions and the need to permeabilize cells expressing these constructs (to allow Cy3NTA entry) limits interpretation of the resulting FRET data. To overcome these problems, we used a dodecapeptide sequence containing a tetracysteine (Tc) motif to target the bi-arsenical fluorophores, FlAsH and ReAsH to RyR1. These compounds freely cross intact cell membranes where they then bind covalently to the tetracysteine motif. First, we used this system to conduct FRET measurements in intact cells by fusing a YFP FRET donor to the N-terminus of RyR1 and then targeting the FRET acceptor, ReAsH to an adjacent Tc tag. High levels of energy transfer (~50%) were observed whereas incubation of ReAsH with a YFP-RyR1 fusion protein lacking the Tc tag resulted in no detectable FRET. We also developed a FRET-based system that did not require GFP fusions into RyR1 by labeling N-terminal Tc-tagged RyR1 with FlAsH, a FRET donor and then targeting the FRET acceptor Cy3NTA to an adjacent His tag. A high degree of energy transfer (~70%) indicated proper binding of both compounds to these unique recognition sequences in RyR1. Thus, these two systems provide unprecedented flexibility in FRET-based structural determinations of RyR1. Supported by NIH grant R01AR059124.

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Structural Mapping of Divergent Region Domains in the Type 1 Ryanodine Receptor using Two Complementary FRET-based Approaches

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We used fluorescence resonance energy transfer (FRET) to localize three divergent region domains within the type 1 ryanodine receptor (RyR1), an intracellular Ca^{2+} channel that mediates skeletal muscle excitation-contraction (EC) coupling. Initial cloning studies of the three RyR isoforms identified three ''divergent regions'' of primary sequence dissimilarity spanning amino acids 4254-4631 (DR1), 1342-1403 (DR2) and 1872-1923 (DR3) in RyR1. These regions have been implicated in EC coupling as well as in differential sensitivity to pharmacological agonists. Here, we used permeabilized HEK-293T cells expressing recombinant RyR1 to localize these DRs to the cryo electron microscopic (EM) map of RyR1. First, we measured FRET from a green fluorescent protein (GFP) donor fused to either position 1 or 620 of RyR1, to a FRET acceptor, Cy3NTA, targeted to poly-histidine ''tags'' inserted into DR1 (at position 4429), DR2 (at position1358) or DR3 (at position 1915). While FRET was not detected for His-tagged constructs containing GFP fused at position 1, FRET was observed from GFP fused at position 620 to all 3 His-tagged positions. Second, we targeted a donor to the RyR1 cytoplasmic domain using FKBP12.6 labeled with Alexa Fluor 488, and then measured FRET to Cy3NTA targeted to the His tag sites described above. Donor-FKBPs bound with high-affinity to both recombinant wild type and His-tagged RyRs. FRET was detected from donor conjugated to each of four, well-separated positions on FKBP to Cy3NTA targeted to each divergent region. Since the fused GFPs and FKBP12.6 have already been localized within the cryo EM map of RyR1, we can now triangulate the DR positions to the cryo EM map from these two complementary data sets. Supported by NIH grant R01AR059124 (to JDF, MM, and TG) and R01HL092097 (to RLC).

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The General Anaesthetic Binding Site of Calmodulin Disrupts Ryanodine Peptide Binding

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The skeletal muscle Ryanodine Receptor (RyR1) is a large calcium release channel involved in excitation-contraction coupling. It is also the target for hundreds of disease mutations that cause malignant hyperthermia (MH) or skeletal muscle disorders like central core disease (CCD). MH is typically triggered by volatile anesthetics, but their binding site on RyR1 has not been fully described. RyR1 is under the control of several auxiliary proteins. One of these is Calmodulin (CaM), a Ca^{2+}-binding protein that can suppress RyR1 activity at elevated Ca^{2+} concentrations. Here we investigate how CaM can bind to RyR1, and how this may be affected by volatile anesthetics. We found that CaM can bind to at least three different RyR1 peptides, with the affinity and lobe specificity being altered substantially by the Ca^{2+} concentrations. In addition, we identified two binding sites for sevoflurane, a volatile anesthetic, on Ca^{2+}/CaM. The anesthetic binds to a pocket that is involved in binding RyR1 peptides. In addition, it can alter the affinity of the N-terminal CaM lobe for Ca^{2+}. These findings suggest that binding of anesthetics to CaM may be involved in the pathophysiology of malignant hyperthermia.

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The Cytoplasmic Foot of RyR1 without the Membrane Spanning Domain Targets Junctionally and Retrogradely Enhances DHPR L-Type Ca^{2+} Currents

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In skeletal muscle, RyR1 (5,037 residues) forms a homo-tetrameric Ca^{2+}-release channel in the sarcoplasmic reticulum (SR), mediates excitation-contraction coupling in response to an orthograde signal from the DHPR in the plasma membrane, and retrogradely enhances L-type Ca^{2+} current via the DHPR. The RyR1 C-terminus forms the Ca^{2+} channel pore across the SR membrane and is believed to be important for inter-subunit interactions, whereas the bulk (~85%) of the protein (the so-called foot) bridges the junctional, myoplasmic gap between the SR and plasma membranes. Here, we have examined the ability of the foot domain (residues 1-4300; $RyR1_{1:4300}$) to target junctionally and interact with the DHPR by expression of a cDNA encoding YFP-$RyR1_{1:4300}$. In dysgenic (α_{1S}-null) myotubes which lack DHPRs, YFP-$RyR1_{1:4300}$ was diffusely distributed and, on the basis of photobleaching, freely mobile within the cytoplasm, consistent with the loss of membrane anchoring. However, after expression in dyspedic (RyR1 null) myotubes (which have DHPRs), much of YFP-$RyR1_{1:4300}$ was immobile within fluorescent foci near the myotube surface, suggestive of junctional targeting and binding to DHPRs. Junctional targeting was confirmed by partial co-localization of YFP-$RyR1_{1:4300}$ and CFP-labeled α_{1S} after co-expression in dyspedic myotubes. Strikingly, YFP-$RyR1_{1:4300}$ was able to retrogradely enhance peak Ca^{2+} current in dyspedic myotubes from 1.6 pA/pF (control) to 6.7 pA/pF, similar to that after expression of full-length RyR1 (7.4 pA/pF). Thus, the isolated, cytoplasmic foot of RyR1 retains the ability to target junctionally and to interact functionally with the DHPR.

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The Cytoplasmic Foot of RyR1 forms a Stable Homotetrameric Structure Despite Lacking the Membrane-Spanning C-Terminal Domains

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In skeletal muscle, the dihydropyridine receptor (DHPR) in the plasma membrane engages in bi-directional interactions with the type 1 ryanodine receptor (RyR1) in the sarcoplasmic reticulum (SR) such that an ''orthograde'' signal from the DHPR triggers SR Ca^{2+} release via RyR1, and a retrograde signal